Aspartic acid racemization variability in ancient human remains: implications in the prediction of ancient DNA recovery

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A B S T R A C T

The extent of racemization of aspartic acid (Asp) – expressed as D/L ratio – has been used as a marker of biomolecular degradation in ancient remains. However, Asp racemization rate is highly variable, and depends on biochemical and geochemical factors. In this paper we aim to determine to which extent the fraction analyzed and the kind of sample used may influence the D/L Asp ratios. Other factors, such as burial site and sample preservation conditions, are also considered.

D/L Asp ratios were obtained in 38 ancient human samples from 30 different individuals in which *Real Time PCR* quantification and amplification of short mtDNA fragments had been previously achieved. Four samples were taken from bones, 16 from whole teeth and 18 from dentine. In 7 cases whole tooth and dentine fractions from the same individual were analyzed. The samples belonged to 8 archaeological sites from Pre-pottery Neolithic B (PPNB), Cardial Neolithic and Chalcolithic time periods. Results show significant differences between the D/L Asp ratios obtained in dentine and whole tooth fractions from the same tooth and individual, as well as among dentine samples from the same archaeological site and among samples of the same age from different, though nearby, archaeological sites. Ancient DNA (aDNA) could be characterized in the majority of the samples, independently from their racemization values, which suggests that other factors, apart from the sample preservation stage, are more significantly affecting the racemization rate. A generalized use of the Asp racemization ratio as a threshold value for ancient DNA preservation needs to be questioned until further methodological standardization is considered.

1. Introduction

All amino acids with at least one chiral carbon center can exist in two stereoisomeric forms, named L (laevorotatory) and D (dextrorotatory). However, only L amino acids are used in protein biosynthesis. When an organism dies, a progressive transformation of L into D isoforms occurs in a process called racemization until the equilibrium is reached. Racemization also takes place *in vivo* in tissues with low protein turn-over, such as tooth dentine, dental enamel, vertebral discs or ocular lens (Helfman and Bada, 1975).

Amino acid racemization reaction is a first order kinetics reaction:

L – Asp ↔ D – Asp

From this expression, it has been defined empirically that the amino acid racemization rate follows an Arrhenius-type equation:

$$\ln(k) = \ln(A) - \frac{E_a}{RT}$$

in which k is the rate constant, A the frequency factor, E_a the activation energy, R the gas constant and T the temperature (Dungworth et al., 1974). If the temperature is held constant, the D/L values are expected to linearly correlate with age. Because of this property, amino acid racemization has been used as a dating tool in fossil mollusc shells, bones and teeth (Bada and Protsch, 1973; Miller et al., 1992). In this last case, a constant depositional temperature must be assumed (Canoira et al., 2003).

The linearity of the amino acid racemization rate has also been demonstrated *in vivo* in tissues with low metabolic activity, and it has been successfully employed in Forensic Medicine for estimating the age at death (Ohtani et al., 1990).

The racemization of aspartic acid (Asp) has been used to assess the extent of DNA decomposition in fossil remains. This amino acid

has one of the fastest racemization rates, and its rate constant and activation energy are very similar to those for DNA depurination, the main reaction responsible for the spontaneous degradation of DNA (Bada et al., 1994; Lindahl and Nyberg, 1972). Poinar et al. (1996) estimated D/L Asp ratios in archaeological specimens from which DNA sequences have been recovered, concluding that no reproducible DNA sequences could be retrieved from samples showing a D/L Asp ratio higher than 0.1. The same authors also showed that racemization ratios of alanine (Ala) or leucine (Leu) could be employed as recent contamination indicators, since the racemization relationship $D/L_{Asp} > D/L_{Ala} > D/L_{Leu}$ should be maintained if all amino acids are endogenous to the sample.

Previous research on modern samples have reported that various factors may influence Asp racemization ratios in human dentine, such as the type of tooth studied within a single individual (Ohtani et al., 2003), the sampling region (Arany et al., 2004; Ritz et al., 1993) or the protein fraction analyzed (Ohtani and Yamamoto, 1990a).

However, the influence of *post-mortem* conditions and site taphonomy on the racemization ratios have been scarcely studied. Some factors, such as pH, ionic strength, metal ions presence and water content, have a direct effect on racemization rates (Bada, 1972). The variability in racemization ratios in samples from the same site has been interpreted as a consequence of the existence of individual differences in age at death (Sinibaldi et al., 1999), differences in response to the extraction procedure (Carolan et al., 1997), and local differences in time of formation of cave layers (Torres et al., 2002).

In this paper we show to which extent the type of tissue, the analyzed fraction and the site taphonomy are responsible for Asp racemization variability in ancient samples. In the light of the results obtained, we also discuss the reliability of Asp racemization as a screening method of aDNA preservation.

2. Materials and methods

2.1. Sample preparation

The studied material consisted of 30 individuals from 8 archaeological sites of different chronology, ranging from Neolithic PPNB to Chalcolithic times (Table 1, Fig. 1). These individuals were selected in terms of their macroscopic preservation state.

Table 1
D/L ratios of Aspartic acid (Asp), Alanine (Ala) and Leucine (Leu) measured in ancient samples. The number of copies of mtDNA was also estimated using a specific *Real Time PCR* design. In the last column the success in the recovery of 150 bp sequences is shown (Fernández, 2005). Samples that fulfill the following authentication criteria are indicated. [a], Independent replication from the same or different extracts; [b], bacterial cloning of *PCR* products; [c], replication in an independent laboratory.

Sample	Skeleton	Tissue	Kind of tooth	Age at death	Site	Dating	D/L Asp	D/L Ala	D/L Leu	DNA copies/μl	DNA ~150 bp
AB9	AB23C	Tooth	Adult	?	Abauntz	4370–4240 B.P.[d]	0.087	0.01		9.11×10^4	Yes
AB14	AB17C	Tooth	Adult	?	Abauntz	4370–4240 B.P.[d]	0.088			3.35×10^4	Yes
AB17	AB35E	Tooth	Adult	?	Abauntz	4370–4240 B.P.[d]	0.155			9.29×10^4	Yes[a]
TM5	TM8	Tooth	Adult	?	Tres Montes	4130 B.P.[e]	0.551	0.20		5.78×10^4	Yes
TM5	TM8	Dentine	Adult	?	Tres Montes	4130 B.P.[e]	0.123	0.05			
TM6	TM11	Tooth	Adult	?	Tres Montes	4130 B.P.[e]	0.121			4.07×10^4	Yes[b]
TM6	TM11	Dentine	Adult	?	Tres Montes	4130 B.P.[e]	0.122				
1MA1	MA1	Tooth	Deciduous	?	Mari	2900–2700 B.C.[f]	0.134	0.02		3.98×10^4	Yes
1MA8	MA8	Tooth	Adult	?	Mari	2900–2700 B.C.[f]	0.126	0.01	0.01	7.93×10^4	Yes
1MA12	MA9	Tooth	Adult	?	Mari	2550 B.C.[f]	0.114	0.02		8.14×10^4	Yes[b]
5NE	NE-1829	Bone		?	Nerja	5785 ± 80 B.P.[g]	0.272	0.08		1.64×10^4	Yes
CA7	O10 107	Tooth	Adult	?	Caldeirao	5321–5732 cal B.C.[h]	0.107	0.00	0.01	3.21×10^4	Yes
TR9-1	R65-4II	Tooth		?	Tell Ramad	5950–6260 cal B.C.[i]	0.120			4.42×10^4	Yes[a,b,c]
TR9-2	R65-4II	Tooth	Adult	?	Tell Ramad	5950–6260 cal B.C.[i]	0.146			5.83×10^4	Yes[a,b,c]
TR16	R65-C8-SEB	Bone		?	Tell Ramad	5950–6260 cal B.C.[i]	0.205	0.14		1.39×10^5	Yes[a,b,c]
TR18	R65-1S	Bone		?	Tell Ramad	5950–6260 cal B.C.[i]	0.212	0.05	0.06	2.03×10^J	Yes[a,b,c]
3DJ6	SK-R3	Bone		?	Dja'de El Mughara	9250 B.P.[j]	0.123			3.60×10^4	Yes
2H25	H25	Dentine	Germ	2	Tell Halula	8700 ± 60 B.P.[k]	0.185				Yes[a,b,c]
1H26	H26	Dentine	Adult	14–16	Tell Halula	8700 ± 60 B.P.[k]	0.108				No
1H30	H30	Dentine	Adult	>30	Tell Halula	8700 ± 60 B.P.[k]	0.224	0.00	0.02		Yes
1H1	H35	Tooth	Adult	>40	Tell Halula	8700 ± 60 B.P.[k]	0.340				No
1H1	H35	Dentine	Adult	>40	Tell Halula	8700 ± 60 B.P.[k]	0.125				
2H20	H37	Tooth	Adult	14	Tell Halula	8700 ± 60 B.P.[k]	0.376	0.23		6.80×10^4	Yes[b]
1H20	H37	Dentine	Adult	14	Tell Halula	8700 ± 60 B.P.[k]	0.244				
2H31	H43	Tooth	Deciduous	6–7	Tell Halula	8700 ± 60 B.P.[k]	0.187				Yes
2H31	H43	Dentine	Deciduous	6–7	Tell Halula	8700 ± 60 B.P.[k]	0.117	0.01			
2H18	H47	Dentine	Germ	0.75	Tell Halula	8700 ± 60 B.P.[k]	0.255			8.61×10^4	Yes
1H33	H48	Dentine	Adult	20–25	Tell Halula	8700 ± 60 B.P.[k]	0.154				Yes
2H15	H49	Dentine	Adult	18	Tell Halula	8700 ± 60 B.P.[k]	0.125			7.14×10^4	Yes
1H19	H52	Dentine	Germ	1	Tell Halula	8700 ± 60 B.P.[k]	0.275				No
2H14	H53	Dentine	?	10	Tell Halula	8700 ± 60 B.P.[k]	0.190			7.63×10^4	Yes[a,b,c]
2H34	H54	Dentine	Deciduous	4	Tell Halula	8700 ± 60 B.P.[k]	0.227				Yes
1H21	H57	Tooth	Adult	25	Tell Halula	8700 ± 60 B.P.[k]	0.141			4.39×10^4	Yes
2H21	H57	Dentine	Adult	25	Tell Halula	8700 ± 60 B.P.[k]	0.203				
1H13	H64	Dentine	Adult	18	Tell Halula	8700 ± 60 B.P.[k]	0.254	0.11	0.09	7.57×10^4	Yes
1H10	H65	Dentine	Adult	>40	Tell Halula	8700 ± 60 B.P.[k]	0.219		0.11	2.39×10^5	No
2H11	H70	Tooth	Adult	20	Tell Halula	8700 ± 60 B.P.[k]	0.179	0.02		1.51×10^5	Yes[a,b,c]
1H11	H70	Dentine	Adult	20	Tell Halula	8700 ± 60 B.P.[k]	0.171				

[d] Utrilla and Mazo (1994).
[e] Andrés et al. (1997).
[f] Margueron et al. (2007).
[g] Cortés et al. (2006).
[h] Zilhão (1993).
[i] Ferembach (1970).
[j] Coqueugniot (1998).
[k] Molist (1996).

Fig. 1. Location of the studied archaeological sites. ● Cardial Neolithic sites; ▲ pre-pottery Neolithic B (PPNB) sites; ■ Chalcolithic/Sumerian sites. (1) Caldeirão; (2) Abauntz; (3) Tres Montes; (4) Nerja; (5): Tell Halula; (6) Tell Ramad; (7) Dja'de El Mughara; (8) Mari.

Moreover, most of them had provided positive amplifications of short mtDNA fragments and high quantification values by *Real Time PCR*. Amino acid content and racemization ratios were estimated in 3 different powdered tissues: 4 samples were taken from bones, 16 from whole teeth, and 18 from the dentine fraction. In 7 cases whole tooth and dentine fractions from the same individual were analyzed. Decidual and definitive teeth were included in the analysis (Table 1).

Bone and whole tooth samples were superficially cleaned using a Sand Blaster (Dentalfarm Base 1 Plus) and then powdered in a Freezer Mill (Millex SPEX 6700) filled with liquid nitrogen. Dentine samples were obtained by drilling a small hole near the tooth crown neck in order to reach the inner part of the crown that was protected by the enamel, allowing the recovery of 20–40 mg of dentine powder from each sample.

2.2. Amino acid racemization

The powdered samples were first dissolved in 1 ml 2 N HCl and then sonicated. To remove free amino acids and to extract collagen fraction, samples were dissolved in 5 ml of phosphate-buffer saline (PBS) and then dialyzed using a dialysis membrane (Spectra/Por mnco 3500D) for a period of 24 h in a buffered solution with magnetic stirring at room temperature (Marzin, 1990). Then, The samples were prepared according to the method of Goodfriend and Meyer (1991). The dialyzed fraction was first hydrolyzed under N_2 atmosphere with 6 M HCl for 20 h at 100 °C and then desalted with concentrated HF. The first derivatization step consisted on the sterification with pure thyonil chloride in isopropanol and the second on the *N*-trifluoroacetilation with pure trifluoroacetic acid anhydride. The derivatized samples were injected into a Hewlett Packard 5890 gas chromatograph with a Chirasil L-Val column. The detection was done by a NPD detector at 300 °C and the PEAK96 HP software was used to integrate the peak areas. The induced Asp racemization background of the procedure used was estimated in 0.04–0.05, much lower than the actual values measured on recent dentine samples (Torres et al., 2002).

2.3. Ancient DNA extraction

Whenever possible, a second sample of the same individual was taken for independent extraction. Approximately 600 mg of bone or whole tooth powder were washed with 0.5 M EDTA pH 8.0 and incubated over-night at 37 °C in lysis buffer solution (5 mM EDTA, 10 mM TRIS, 0.5% SDS, 50 μg/ml Proteinase K). Tissue remains were removed by centrifugation and DNA was extracted from the supernatant by a modified Phenol/Chloroform protocol and concentrated with Centriplus-30000 micro-concentrators (Millipore) (Fernández et al., 2006). Extraction controls without powdered sample were processed in parallel to test for exogenous DNA contamination during the extraction process.

2.4. mtDNA Real Time PCR quantification

A *Taq-Man Real Time* assay was used for the specific quantification of mitochondrial DNA (mtDNA) Hypervariable Region I (HVRI) (positions 16103–16233) in the obtained extracts using *Taq-Man-MGB* probe 5′-AATACTTGACCACCTGTAGTAC-3′ (positions 16138–16159) and primers L16123 (forward) (5′-ACTGCCAGCCAC-CATGAATATT-3′, positions 16103–16123) and H16209 (reverse) (5′-TGGAGTTGCAGTTGATGTGTGA-3′, positions 16209–16233). This method was designed to quantify low-copy number extracts (i.e. less than 0.1 ng DNA according to Gill et al., 2000) within the range of 10^9–10^3 copies (3.58–3.58×10^{-6} ng/μl).

PCR reactions were performed using *TaqMan Universal PCR Master Mix* (Applied Biosystems). Samples were loaded onto a standard 96-Well Reaction Plate (Applied Biosystems) and fluorescence detection was performed in a Sequence Detection System ABI Prism 7700 (Applied Biosystems). Four negative controls were included per plate. DNA concentrations from extracts were derived from comparison with a known concentration of cloned extant human mtDNA standard. All extracts were quantified twice and average values were considered.

2.5. mtDNA amplification and sequencing

Two short overlapping fragments of the mtDNA Hypervariable Region I (HVRI) were amplified in the selected samples using a nested-*PCR* approach with primers and conditions described at Fernández et al. (2006). Amplification products were sequenced in an automated sequencer ABI Prism 310 (Applied Biosystems).

2.6. Precautions in the analysis and criteria of authenticity

All extraction and amplification procedures were carried out following strict ancient DNA precautions in order to prevent sample contamination. The studied material was unwashed after the excavation. All analyses were performed in exclusive ancient DNA laboratories in which extraction, preparation of *PCR* reactions and *post-PCR* procedures were physically separated. The access to these laboratories was limited to one researcher (E.F.), who wore clean-room protective clothes, gloves and facemask. The laboratories were routinely cleaned with bleach and UV-radiated. Samples and reagents were manipulated in laminar flow cabinets, which have been previously cleaned with bleach and irradiated with UV light for 30 min. Exclusive material for ancient DNA analysis was used in every experimental process. Before the analysis, all the material employed in the analyses (reagents, tubes, pipettes, racks, filter pipette tips) was gently wiped with bleach, disposed in the cabinet and UV-irradiated for 30 min.

The following criteria of authenticity were employed:

- The obtained sequences were compared to those from the archaeologists, anthropologists and researchers involved in the retrieval or manipulation of the studied samples, and no matches were found (See Supplementary material Table S2).
- Only extracts and amplicons from extraction and amplification groups providing negative results at the blanks were considered.

- To detect carry-over contaminations, sequences from the same or close amplification groups were compared and non-consistent results were discarded.
- When possible, results were independently replicated from the same or different extracts (skeletons AB35E, R65-4II, R65-C8-SEB, R65-1S, H70, H25, H53).
- Amplification products from individuals AB35E, R65-4II, R65-C8-SEB, R65-1S, H25, H53 and H70 were cloned, and 3–15 clones per amplicon were sequenced.
- All extracts were quantified by *Real Time PCR*, providing in all cases a number of copies higher than 1000. As it has been asserted in several other works (Gilbert et al., 2003; Handt et al., 1996) this value is high enough to guarantee the sequence reproducibility.
- Some amplification products were cloned and several clones were sequenced (skeletons AB35E, TM6, MA9, R65-4II, R65-C8-SEB, R65-1S, H37, H70, H25, H53) (See Supplementary material Table S3)
- Some samples were replicated in a second laboratory (skeletons R65-4II, R65-C8-SEB, R65-1S, H70, H25, H53).

3. Results

3.1. Total Asp content

Total Asp content (D + L isoforms) measured in the whole tooth fraction was larger in the Chalcolithic samples than in the Neolithic ones (Fig. 2; Supplementary material Table S1). This pattern could also be observed for the dentine fraction but, in this case, overall amino acid values were much lower than those for whole tooth.

3.2. Racemization ratios

Asp racemization ratios were estimated in all studied samples, but simultaneous measures of D/L racemization for the 3 amino acids (Asp, Leu, Ala) could only be obtained in 5 samples (Table 1), most probably because of the slow racemization rates of Ala and Leu.

The paired comparison of Asp racemization ratios with at least one of the other two amino acids, is consistent with the racemization pattern predicted by Poinar et al. (1996) in the absence of exogenous contamination (D/L Asp > D/L Ala > D/L Leu). Only two samples (TR18, H30) showed D/L ratios for Leu slightly higher than those for Ala, but both values are much lower than the D/L Asp ratio. This fact is related to the aforementioned failure in the detection of D-isoforms in slow-racemizing amino acids in recent samples.

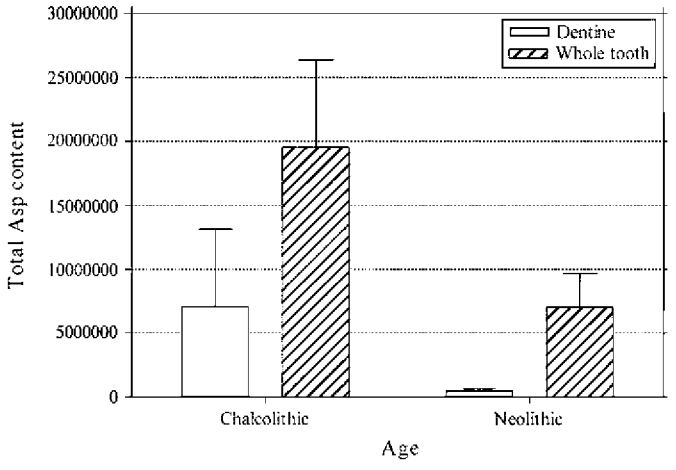

Fig. 2. Average total aspartic acid content of Neolithic and Chalcolithic samples measured in dentine and total tooth fractions. Standard deviation of every group is also shown.

Two different results arise from the comparison of the Asp racemization values between whole tooth and dentine fractions measured in the same individual (skeletons TM8, TM11, H35, H37, H43, H57 and H70). While in some cases the obtained racemization ratios in the two tissues were only slightly different (skeletons TM11, H43 and H70), other samples exhibited very different values (skeletons TM8, H35, H37 and H57). In general, D/L ratios were higher in powdered tooth than in dentine.

When samples are grouped by archaeological site (Table 2) the coefficient of variation by tissue type within each site is considerably variable, ranging from 2 to 118%. This could be both a result of the effect of different environmental conditions in the site over the samples and/or a consequence of the existence of slight differences in chronological age in the same archaeological level. This last possibility was explored by plotting average Asp racemization results for the archaeological site of Tell Halula against archaeological phases. A constant time span of 60–100 years between each phase was assumed (Fig. 3). The correlation coefficient obtained for the linear regression ($R^2 = 0.42$, $r = 0.65$) is not statistically significant ($P = 0.23$). However, the obtained correlation ratio (R^2) indicates a relationship of almost 50%, thus suggesting that chronological differences inside the same archaeological level could be in part responsible of the dispersion in racemization values.

Despite this, the linear relationship between the mean dentine racemization ratios and the chronological age (Fig. 4) shows a highly significant ($P = 0.03$) correlation coefficient ($R^2 = 0.998$, $r = 0.999$), clearly indicating that higher racemization values are obtained in older samples. The same result arises when samples are grouped by chronological age (Table 3).

3.3. Racemization and DNA recovery

As it has been mentioned above, only those samples providing positive amplification results and high mtDNA quantification levels were selected for racemization study. The estimated success rate from the whole sample set was around 20%, it being compatible with other ancient DNA studies.

Only 3 out of the 38 analyzed samples (7.9%) showed Asp racemization ratios falling inside the proposed interval for reliable endogenous DNA recovery (Poinar et al., 1996). The remaining 35 samples exhibited Asp racemization ratios ranging from 0.11 to 0.55. However, *Real Time PCR* quantification showed that 23 of the 35 ancient extracts had over 10^4 DNA copies per μl and mtDNA HVRI sequences could be recovered in 27 of them (Table 1).

4. Discussion

4.1. Differences in tissue and in tissue fractions

The results of this study highlight the existence of several sources responsible for the variability in amino acid racemization ratios in archaeological human remains, the main ones being the type of tissue (bone, tooth) and the tissue fraction analyzed (dentine or whole tooth), both independent of the sample preservation state. Differences in racemization values between bone and tooth also take place *in vivo* (Ohtani, 1998; Ohtani and Yamamoto, 1990b) and must be attributed to the different ultra-structural organization of both tissues, which is in turn responsible for a differential protection against environmental factors. While the enamel protects inner tooth, bone is more porous and consequently more prone to suffer from more rapid biomolecular degradation (Gilbert et al., 2005; Jans et al., 2004). In fact, several studies have reported better DNA yields in teeth than bone (Kurosaki et al., 1993;

Table 2
Asp racemization descriptive statistics by archaeological site. *N*: number of studied samples. SD: standard deviation; VC: variation coefficient.

Site	Age	Tissue	*N*	Average D/L Asp	SD D/L Asp	D/L Asp minimum	D/L Asp maximum	%VC
Abauntz	4370–4240 B.P.	Whole tooth	3	0.106	0.039	0.087	0.155	36.88
Tres Montes	4130 B.P.	Dentine	2	0.122	0.001	0.122	0.123	0.69
Tres Montes	4130 B.P.	Whole tooth	2	0.258	0.304	0.121	0.551	117.81
Mari	2900–2700 B.C.	Whole tooth	3	0.124	0.010	0.114	0.134	7.88
Nerja	5785 ± 80 B.P.	Bone	1	0.272	–	–	–	–
Caldeirao	5321–5732 cal B.C.	Whole tooth	1	0.107	–	–	–	–
Tell Ramad	5950–6260 cal B.C.	Bone	2	0.208	0.005	0.205	0.212	2.26
Tell Ramad	5950–6260 cal B.C.	Whole tooth	2	0.133	0.018	0.120	0.146	13.83
Dja'de El Mughara	9250 B.P.	Bone	1	0.123	–	–	–	–
Tell Halula	8700 ± 60 B.P.	Dentine	17	0.182	0.054	0.108	0.275	29.71
Tell Halula	8700 ± 60 B.P.	Whole tooth	4	0.256	0.102	0.107	0.376	40.02

Oota et al., 1995). However, the differential preservation of other biomolecules like proteins in both tissues has been less studied (Gilbert et al., 2005). In the studied samples, Asp racemization ratios are higher on average in bone than in tooth samples. Nevertheless, the low number of bone samples analyzed and the variety of sites and tissue fractions compared makes the aforementioned hypothesis difficult to test.

The reported differences between complete powdered tooth and dentine fraction in the same individual could be produced by the existence of different racemization rates in each tooth tissue. Complete pulverization of the tooth provides a mixture of tooth tissues (dentine, cementum and enamel), each having a different racemization rate. Whether the enamel and the cementum are complete or partially eliminated with the abrasive treatment previous to powdering is difficult to know. Enamel racemization mainly corresponds to short chain intra-crystalline proteins, as it contains virtually no collagen (Griffin et al., 2008). Therefore, even if not being completely removed, the short peptides would have eluted in the dialysis step, and its effect over the racemization ratios would be expected to be negligible. Racemization rate in cementum, corresponding to collagen proteins, is faster than in dentine, probably due to its outer location in tooth (Ohtani, 1995). The presence of some residual cementum at the total tooth fraction could be in part responsible for the observed differences in racemization ratios when compared to dentine in the same individual.

Regional differences in dentine location should also be taken into account. Due to the pattern of dentine formation, dentine racemization is not homogeneous, being higher in the outer layers of the crown and decreasing gradually to the root (Ritz et al., 1993). Besides, lingual dentine tends to show higher racemization ratios than labial dentine because the lingual part is exposed to higher environmental temperature during life (Ohtani, 1997).

Finally, the observed differences between total tooth and dentine fractions could be the result of differential preservation of crown and root dentine. While the former is protected from the environment by the enamel, the latter is exposed due to the intrinsic porosity of tooth roots (Gilbert et al., 2005).

4.2. *Differences in D/L Asp ratios among and inside archaeological sites*

Differences in chronology could be in part responsible for the observed differences in racemization ratios among archaeological sites, as it can be deduced from Fig. 4 and Table 3. However, this is not the only factor explaining the results, as sites within the same age range and geographic location, such as Tell Halula and Tell Ramad, showed different racemization values for the same tissues, a result from which it is difficult to draw a meaningful conclusion. The high coefficients of variation shown by archaeological sites and tissues (Table 2) are similar to those reported for historical (Carolan et al., 1997; Gilbert et al., 2005; Kolman and Tuross, 2000) and prehistorical human remains (Serre et al., 2004), and also for cave bears (*Ursus deningeri/spelaeus*) (Torres et al., 2002).

The existence of slight differences in chronology inside the same site has yet been explored in Fig. 3. Despite being not significant, a certain relationship between both factors can be deduced.

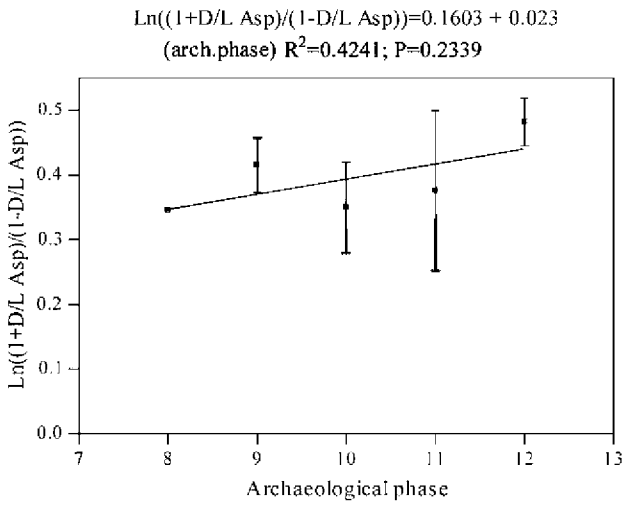

Fig. 3. X–Y plot of aspartic acid racemization ratio average values (±1σ) against archaeological phases, measured in dentine samples from the Neolithic site of Tell Halula.

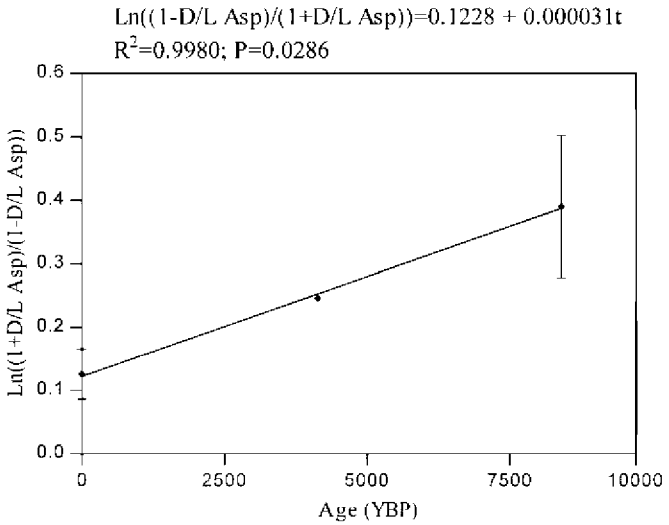

Fig. 4. X–Y plot of aspartic acid racemization ratio average values (±1σ) against radiometrical dates in dentine samples. $X = 0$ represents the average racemization ratios estimated in 23 living individuals using the same method. YBP: Years Before Present.

Table 3
Asp racemization descriptive statistics by age classes and tissue. Chalcolithic: archaeological sites of Abauntz, Tres Montes, Nerja and Mari. Cardial Neolithic: Gruta do Caldeirão; Neolithic PPNB: archaeological sites of Tell Halula, Tell Ramad and Dja'de El Mughara. *N*: number of studied samples. SD: standard deviation; VC: variation coefficient.

Age classes	Tissue	*N*	Average D/L Asp	SD D/L Asp	D/L Asp minimum	D/L Asp maximum	%VC
Chalcolithic	Dentine	2	0.122	0.001	0.122	0.123	0.69
Chalcolithic	Whole tooth	8	0.141	0.155	0.087	0.551	110.09
Chalcolithic	Bone	1	0.272	–	–	–	–
Cardial Neolithic	Whole tooth	1	0.107	–	–	–	–
Neolithic PPNB	Dentine	17	0.182	0.054	0.108	0.275	29.71
Neolithic PPNB	Whole tooth	6	0.205	0.107	0.108	0.275	51.92
Neolithic PPNB	Bone	3	0.175	0.049	0.205	0.212	28.22

In the light of these results, the observed pattern could be interpreted in two ways: as the existence of intrinsic individual differences among the human remains and as a differential response of the samples to taphonomic factors.

One factor to be considered is the age at death of the individuals. In tissues with low protein turn-over, the age of the individual has been demonstrated to correlate with racemization ratios (Ohtani et al., 1990, 1995, 1998; Ritz and Schutz, 1993). Taking this into account, the measured racemization in these tissues in archeological remains might be the sum of *in vivo* and *post-mortem* racemization processes. Depending on the deviation caused by this last component, the dispersion between individuals from the same archaeological bed will be higher or lower. The relationship between ages at death, determined anthropologically ($N = 10$) with corrected racemization ratios in dentine fraction of Tell Halula is non-significant ($\ln[(1 + D/L)/(1 - D/L)] = 0.3675 + 0.00012t$; $R^2 = 0.00016$; $r = 0.0128$; $P = 0.9719$) (Supplementary material Fig. S1). If the same analysis is performed using samples from the same archaeological level, the correlation coefficient clearly increases, but the result is still non-significant ($\ln[(1 + D/L)/(1 - D/L)] = -0.132 + 0.0207t$; $R^2 = 0.8507$; $r = 0.922$; $P = 0.2525$) (Supplementary material Fig. S2). Though this last data should be interpreted with caution because of the reduced sample size tested ($N = 3$), the results obtained suggest that the relationship between age at death and racemization is maintained even after prolonged burial of the samples.

Differences in sample ultra-structure (porosity, presence of micro-fissures) might cause a differential response to the burial environment resulting in racemization disparity in the same site (Reiche et al., 2003). Although all selected dental pieces were well preserved and without visible fractures, tooth ultra-structure was not explored. One factor that was thought to account for differential exposure to burial environment was the kind of tooth analyzed (deciduous or definitive). In the present work deciduous teeth tend to show higher ratios than definitive teeth (Table 1), but when tested statistically this difference is not significant (unpaired *t* test; $N = 7$, $t = 1.132$, 14 d.f., $P = 0.2766$), probably due to the reduced sample size analyzed. One explanation for this phenomenon could be that enamel is significantly thinner in deciduous than in permanent teeth (Grine, 2005), thus providing a less effective protective effect against the environment in the former than in the latter.

Environmental conditions inside an archaeological site are not homogeneous (Torres et al., 2002), so depending on its location a fossil will undergo variable temperature, pH, water lodging, sediment composition, etc (Reiche et al., 2003). Taking this into account, the preservation state of the remains will be a combination of its location in a site and the aforementioned differences in fossil ultra-structure.

Finally, the reported dispersion in Asp racemization ratios in dentine samples from the same site and archaeological level seems to be compatible with the Asp kinetics model in bound collagen for archaeological samples proposed by Collins et al. (1999). This model assumes that racemization inside the collagen triple helix does not take place under collagen melting temperature (68 °C in demineralized collagen and 150 °C in mineralized collagen). Therefore, the increase in racemization in mineralized collagen may correspond to terminal collagen residues and to the non-collagenous proteins (NCPs) attached to the triple helix. In archaeological collagen measured Asx (aspartic acid + asparagine) racemization depends on the relative proportion of denatured and triple-helix collagen, which in turn hinges on the denaturation and leaching collagen rates. Under this model, erratic Asp ratios could be obtained independently on the sample preservation stage, depending on the fraction analyzed and the degree of leaching of denatured collagen (Collins et al., 1999).

On the contrary, Llamas et al. (1999) found that, in kinetic experiments on bear dentine (mineralized collagen), measured racemization was produced at temperatures largely below the denaturation point. This model does not explain either the reported linear increase in racemization with time in ancient bear dentine samples measured with the same method and in the same fraction (Torres et al., 2001, 2002), as also here in Fig. 4. Alternatively, A mixed model, with a fast erratic racemization component, accounting for the dispersion in racemization ratios within the same site, and a slow-racemization one, responsible for the increase in racemization with time–, would be consistent with the observed data. Racemization measured in the present research corresponds to 3500 Da dialyzed fraction, which might include insoluble collagen fraction (IC) and also probably high molecular weight soluble peptide fraction (SP). The relative proportion of each fraction and its contribution to total aspartic racemization is impossible to assess with present data. In the proposed model, the erratic component could correspond to soluble peptides >3500 and the time-dependent racemization should occur at high-molecular weight (HMW) fraction. Racemization at HMW fraction in palaeontological samples and its relation to chronological age has been reported by other authors (El Mansouri et al., 1996; Brock et al., 2007).

Whether this racemization occurs inside the triple helix, or corresponds to the telopeptides, NCPs or to the SP attached to the IC by condensation reactions (Carolan et al., 1997; Collins et al., 1992), needs to be evaluated in further studies.

4.3. Assessment for DNA preservation

We have found no correlation between Asp racemization and the likelihood in DNA recovery. Furthermore, we succeeded in obtaining endogenous DNA sequences from samples with dentine racemization ratios ranging from 0.11 to 0.551.

Since it was first published in 1996, the usefulness of the racemization of the Asp as an assessment method to evaluate DNA preservation has not been tested in a large group of human samples. The dispersion of racemization values within a site has been clearly reported, as discussed above, but its influence over the assessment in DNA preservation has never been tested. In the present study we have reported different levels of variability associated to the estimation of aspartic racemization that are not

dependent on sample degradation, such as the type of tissue, the tissue fraction and the kind of tooth analyzed. If not accounted for, this variability may damp the true relationship, if it ever existed, between racemization and DNA preservation.

5. Conclusions

The obtained results show that there are a variety of factors that could affect the racemization rates measured in archaeological samples. Whereas some of these factors could be related to sample preservation, as is the case for chronological age and sample origin, others like the tissue or the sample fraction could not. The assessment of the use of the aspartic acid as a marker for DNA preservation should be achieved only under the decomposition of the different levels of variability and its separate evaluation. The great dispersion in racemization rates detected within the same archaeological site advices against the extrapolation of racemization values found at one single sample to the extent of DNA preservation of the whole site.

A new model taking into account all these factors needs to be developed in order to implement Asp racemization as a reliable tool in the study of ancient biological remains.

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